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(57) Abstract

Treatment of basic fibroblast growth factor (bFGF) with organic disulfides, preferably glutathione disulfide, or with inorganic compounds of similar function results in a bFGF composition of enhanced stability and resistance to multimerization. The resulting stabilized form mimics the chromatographic behavior of bFGF as isolated from bovine pituitary.

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IMPROVED FORMS OF BASIC FIBROBLAST GROWTH FACTOR

Technical Field

The invention relates to the field of stabilizing protein compositions. More specifically, it concerns procedures for stabilizing basic fibroblast growth factor by treatment with disulfides or other compounds capable of forming S-S covalent bonds.

Background Art

One of the persistent problems involved in the recombinant production of desired proteins resides in the obtainment of proper processing and folding of the recombinant product. Because the gene encoding the primary protein structure is generally expressed in a host system foreign to that in which it is normally transcribed and translated, the resultant product protein, while having the correct amino acid sequence, has different characteristics from the native protein. In some instances, a part of this difference resides in a change in molecular structure--most commonly, lack of normally associated glycosylation. However, it is also understood that the three-dimensional conformation of the protein may be different depending on the cellular environment. In particular, various recombinant hosts are thought to have differing environments with respect to the level of oxidation or reduction that normally is associated with the cellular components, resulting in recombinant proteins with disulfide bond compositions which differ from those of the native protein, and, in general, altered three-

dimensional structures. These alterations may have deleterious effects on behavior and activity.

In the case of basic fibroblast growth factor (bFGF), glycosylation patterns are not germane as the native molecule is unglycosylated. However, recombinantly produced material differs from that isolated from pituitaries in its chromatographic behavior and poses stability problems unless measures are taken to prevent multimerization in solution. It has been shown, by applicant herein, and as disclosed by Seno, M., et al., Biochem Biophys Res Comm (1988) 151:701, that substitution of serine for the cysteines at positions 78 and 96 prevents multimerization. It is also the case that bFGF isolated from natural bovine pituitary does not multimerize.

It has been disclosed that amino acid composition determinations of native isolated bovine bFGF indicate the presence of six cysteines, although the gene encodes only four. It was proposed that additional residues of cysteine might be disulfide linked to the protein (Esch, F., et al., Proc Natl Acad Sci USA (1985) 82:6507).

The formation of conjugates of cysteine or glutathione with proteins by reaction with cystine or glutathione disulfide as a natural phenomenon has long been suggested and disclosed. In 1960, Eagle, H. et al., J Biol Chem (1960) 235:1719-1726 showed that mammalian cell cultures were abetted by addition of compounds capable of forming S-S covalent bonds, including, e.g., cysteine, $S_2O_3^{-2}$, and thioglycolate. The authors concluded that the proteins in the medium were bound by S-S bonds to these reagents. Human serum albumin was disclosed to exist in a dimerized form, presumably disulfide bonded, and in a monomeric form stabilized by cysteine or glutathione by King, T.P., J Biol Chem (1961) 236:PC5. Binding to glutathione was proposed to account

for the heterogeneity of hemoglobin by Huisman, T.H. et al., J Lab & Clin Med (1962) 60:302-319.

Hanap, K.R. et al., Biochim Biophys Acta (1973) 310:104-110 acknowledged the presence of mixed disulfides of glutathione and serum albumin and found them reducible by glutathione reductase. Isaacs, J. et al., Biochim Biophys Acta (1977) 497:192-204 propose formation of mixed disulfides of proteins with glutathione as a means to regulate S-S/SH ratio. Conversely, Mannervitc, B. et al., Biochim J (1980) 190:125-130 postulate the formation of disulfides with proteins to represent a mechanism for regulation of protein activity. Pyruvate kinase is used as an example.

It has also been proposed, because of the conservation of cysteines at residues 34 and 101 in the genes encoding bFGF in various mammals, that an intramolecular disulfide is formed between these two residues, and the formation of this disulfide in the recombinant mutant form of bFGF having serine substituted for cysteine at positions 78 and 96 has been reported (Fox, G.M., et al., J Biol Chem (1988) 263:18452).

It has now been found that addition of an organic disulfide-containing compound, such as glutathione disulfide, to preparations of recombinantly produced bFGF results in enhanced stabilization and behavior more closely related to the native protein. Purification of this stabilized form of bFGF results in a preparation suitable for formulation for pharmaceutical applications.

Disclosure of the Invention

The invention provides basic fibroblast growth factor (bFGF) in a form which is stable, convenient, similar to the native protein in activity, and adaptable to pharmaceutical formulations. This form is obtained by treating a susceptible form of basic fibroblast growth factor or its analogs with a protective agent capable of

forming a covalent S-S bond with bFGF, such as an organic disulfide, e.g. glutathione disulfide (GSSG). The thus-stabilized bFGF can then be further purified using standard chromatographic techniques for use in pharmaceutical or other compositions.

Thus, in one aspect, the invention is directed to a method to stabilize bFGF protein which method comprises treating a susceptible peptide having the amino acid sequence of bFGF with an amount of a protective agent capable of forming a covalent S-S bond with bFGF, preferably a compound of the formula RSSR wherein R is an organic moiety, effective to prevent multimerization, in the presence of a buffer of pH 6 or greater, and at which the bFGF does not denature, for a time sufficient to effect said stabilization.

In other aspects, the invention is directed to bFGF prepared according to the methods of the invention and to the compositions useful in the invention method.

Brief Description of the Drawings

Figure 1 shows traces from reverse phase HPLC conducted on samples of recombinant bFGF and its recombinant C78/96S analog with or without treatment with glutathione disulfide (GSSG).

Figure 2 shows traces of reverse phase HPLC conducted on GSSG-treated recombinant bFGF and bovine pituitary bFGF.

Figure 3 shows the activity profiles for untreated recombinant bFGF and for bFGF treated with GSSG.

Figure 4 shows a comparison of results of heparin-TSK HPLC chromatography of recombinant bFGF, which is untreated or treated with GSSG, and of bovine pituitary bFGF with or without prior treatment with cupric ion (Cu^{+2}).

Figure 5 shows the results of a series of heparin TSK HPLC chromatographies on bFGF untreated and treated with GSSG over a 5 day time period.

Figure 6 shows the results of reverse phase HPLC conducted on recombinant bFGF treated with cystine, as compared to native bovine pituitary bFGF.

Figure 7 shows the results of reverse phase HPLC conducted on recombinant bFGF analogs with and without prior treatment with GSSG.

Figures 8 and 9 give the DNA coding sequences for, and deduced amino acid sequences of, bovine and human basic FGF, respectively.

Modes of Carrying Out the Invention

As used herein, basic fibroblast growth factor is defined as a protein which is a cation at pH 7 and which is capable of mitogenic activity in in vitro assays using cultured cells, such as bovine brain and adrenal cortex-derived capillary endothelial (ACE) cells, human umbilical vein endothelial cells, bovine adrenal cortex cells, granulosa cells, or vascular smooth muscular cells. In vitro assays employing these cell cultures have been described by Gospodarowicz, D. et al., J Cell Physiol (1985) 122:323-332, Gospodarowicz, D. et al., J Cell Biol (1983) 97:1677-1685, Esch et al., Proc Natl Acad Sci USA (1985) 82:6507-6511 and Gospodarowicz, D. et al., J Cell Physiol (1986) 127:121-136. In addition, in vivo assays using chicken chorioallantoic membrane have also been described by Gospodarowicz, D. in "Hormonal Protein and Peptides" XII:205-230 (Academic Press). A protein which is a cation at pH 7 and which has activity in at least one of these assays is defined as basic FGF.

The amino acid sequences fulfilling this definition are of various embodiments. Preferred embodiments are shown in Figures 8 and 9, which indicate the amino

acid sequences of the basic FGFs encoded by genes isolated from bovine and human libraries, respectively. These genes encode proteins of the form generally isolated from, e.g., pituitary, having 146 amino acids, beginning with the proline residues numbered "1" below the sequences in the Figures. The indicated human and bovine 146 amino acid sequences are two preferred embodiments of the basic FGF to which the method of the invention is applicable. Also preferred are active forms of the proteins shown in Figures 8 and 9 which include N-terminal extensions of the 146 amino acid sequence, as there shown, as well as N-terminal shortened forms of these sequences lacking as many as 15 amino acids from the 146 amino acid sequence. Particularly preferred are the forms containing all seven or eight upstream amino acids shown exclusive of the N-terminal methionine (which is removed during production of recombinant bFGF in bacteria), for a total of 153 or 154 amino acids, and a shortened form lacking the first 15 amino acids of this basic sequence, containing 131 amino acids. When the protein is produced recombinantly, the 154 amino acid form results from expression of the complete 155 amino acid sequence which is followed by removal of the Met by post-translational processing. Most preferred are the 153 or 154 amino acid forms beginning with the ala residues shown as positions 2 or 3 in the numbering above the sequences, or mixtures thereof. In general, the numbering system used herein is that corresponding to the overplaced numbers.

Most, but not all, proteins which fall under the definition of basic FGF, besides showing FGF activity in at least one of the foregoing assays and being cations at neutral pH, bind to heparin and react immunologically with antibodies prepared using a synthetic analog of the amino terminal sequence or to other antibodies raised against bovine or human basic FGF or synthetic or native peptide fragments thereof, as appropriate. Generally, these

proteins are at least 80% homologous with the bFGF proteins of Figures 8 or 9, preferably 90% homologous. Among preferred embodiments are analogs of bFGF which retain FGF activity in the foregoing assays, such as those described in U.S. Serial No. 070,797, filed 7 July 1987 and incorporated herein by reference. In order to be a suitable substrate in the method of the invention, the bFGF should include cysteine capable of forming a covalent -S-S- bond, e.g. one, and preferably two cysteine residues in positions other than those involved in the (putative intramolecular disulfide linking) positions 34 and 101.

In general, the method of the invention is applicable to any "susceptible" bFGF protein, which forms disulfide bonded multimers in the absence of antioxidants. The propensity of a protein to form multimers can be assessed readily by, for example, subjecting a preparation of the protein which is exposed to air for various times to standard high performance chromatography methods to detect the presence of higher molecular weight forms. Alternatively, multimerization can be detected by subjecting these preparations to SDS-PAGE under reducing and nonreducing conditions. The results under reducing conditions provide the standard nonmultimerized molecular weight; nonreduced materials will show the presence of multimers. For proteins which multimerize under this regimen, stabilization of the nonmultimerized form can be obtained using the method of the invention.

Usually, proteins which multimerize contain at least one cysteine residue which is not involved in intramolecular disulfide linkages. Of course, it is not possible, by simply assessing the number of cysteines in the amino acid sequence, to discern whether the protein in question is susceptible to stabilization by this method, since examination of the sequence does not permit prediction of the extent to which intramolecular disulfides are formed. However, it has been shown by the inventors

herein that, for example, acidic FGF, which has some homology to basic FGF, has similar activity, and is an anion at neutral pH, does not multimerize, nor does it react with GSSG. This shows that "susceptibility" is not obvious from amino acid sequence but must be determined experimentally. Acidic FGF has an odd number of cysteines, so that formation of intramolecular disulfide leaves one free cysteine, and the protein is capable, therefore, of dimerization.

It is appropriate, therefore, to define the meaning of "susceptible" bFGF proteins--these are bFGF proteins which can be stabilized by the method of the invention, as shown by the assay described above. In the case of basic FGF, it is required, for susceptibility, that at least one of the cysteines at position 78 and/or 96 as shown in the overlined numbers of Figures 8 and 9 (or a cysteine corresponding to these, e.g., at a similar position in an analog) be present. It is more preferred that both cysteines be present. In order to maintain susceptibility, the precise positioning of these residues at the locations found in the native sequence is not necessary, and, in general, susceptible forms for basic FGF are those which have at least one, and preferably two cysteine residues available for multimerization, i.e. not contained, under the relevant conditions, in intramolecular disulfide bonds.

It is further noted that "native" basic FGF isolated from mammalian tissues is, under most published isolation protocols, already stabilized, presumably by indigenous glutathione disulfide available in situ. Basic FGF is susceptible to treatment to prevent multimerization only when produced under circumstances where this native stabilization does not occur. This occurs when an isolation protocol includes treatment of proteins with a reducing agent or most commonly in the recombinant production of basic FGF, especially in procaryotic systems, or in

those eucaryotic systems which do not provide the correct environment for indigenous stabilization.

The Protective Agent

In general, the protective agents are reagents which are capable of forming a covalent S-S bond with a cysteine residue in the subject protein. Most commonly, the bond formed will be a disulfide bond, and the protecting agent will be of the formula RSSR, wherein each R is independently an organic residue which is sufficiently hydrophilic to permit water solubility. The most convenient embodiments of RSSR are those wherein both R residues are identical, as these are readily available in many instances and/or easily prepared from the component sulfhydryl compounds. For example, cystine, homocystine, cystamine, and glutathione disulfide (GSSG) are commonly available reagents. Mixed disulfides of these components could also be used, although there is no particular point in using the mixed disulfide. Also readily prepared, but very expensive, is the disulfide of coenzyme A.

It is seen from the foregoing exemplary compounds of the formula RSSR, that the nature of R is generally a hydrocarbyl with sufficient polar substitutions to permit water solubility. Thus, preferred embodiments for RSSR are those wherein the sulfur atom residue is a part of an amino acid or peptide, as in GSSG or cystine.

In addition to protective agents of the formula RSSR, inorganic compounds capable of forming a covalent S-S bond can be used. A convenient inorganic reagent is the salt of tetrathionate ion, i.e., $S_4O_6^{-2}$. In this case, the stabilized form of the subject peptide will have a derivatized cysteine with a substituent of the formula $-S-SO_3^-$. Also usable as a protective agent is a mixed reagent of the formula $RS-SO_3^-$.

In summary, the protective agent can be any moiety which forms a covalent S-S bond with the sulfhydryl of a free cysteine in the substrate protein to be stabilized. Preferred in the stabilization of proteins intended for pharmaceutical use are reagents which generate pharmaceutically acceptable derivatives; therefore, use of compounds which derivatize the cysteine of the substrate with biologically compatible substances such as glutathione or cysteine are preferred.

General Method

The stabilization of the subject susceptible protein is conducted in a buffered aqueous solution. The pH may range between 6 and whatever upper limit is dictated by the stability of the protein with respect to denaturation. Any commonly employed buffer system which does not contain free sulfhydryl groups or thiol binding agents can be used, including phosphate buffer, Tris, and so forth. It may be desirable to add small concentrations of EDTA, about 1 mM, in order to prevent side reactions. The protein is dissolved in the buffer at a convenient concentration, depending on the solubility of the protein. Typical concentrations are in the range of 0.1-10 mg/ml. The protecting agent is employed at any convenient concentration, determined in part by the concentration of the subject protein employed. As the reaction is desired to proceed to completion, concentrations of reagent which result in a 10- to 100-fold molar excess over the amount of protein are desirable. Suitable amounts under many conditions of protein solute concentration are 0.1 mM-100 mM, or more usually 0.5-5 mM, depending on the solubility of the reagent. The reaction mixture is incubated for a time effective to confer stability, typically for a few hours to overnight, at a temperature of about $4^{\circ}C$ to about room temperature. The resulting stabilized protein is then purified using standard techniques. Depending on the

choice of protecting agent, the protein is generally the only macromolecular substance in solution, and is thus easily recovered by gel filtration; other standard techniques may be used further to purify the protein. For example, if basic FGF is the subject protein, heparin sepharose affinity chromatography is conveniently used, as described by Gospodarowicz, D. et al., Proc Natl Acad Sci USA (1984) 81:6963.

The following examples are intended to illustrate, but not to limit, the invention.

Preparation A

Preparation of Recombinant bFGF

Human basic FGF was produced recombinantly in E. coli in a manner similar to that described in copending application U.S. Serial No. 869,362, filed 30 August 1986, and incorporated herein by reference. The bFGF was recovered from the cell lysate and purified.

Briefly, and in particular, the human bFGF gene used encodes a 154 amino acid form of the protein beginning with the N-terminal sequence MetAlaGlySerIle. This bFGF gene has the sequence shown in Figure 9, except that the ala at position 2 is deleted. When produced recombinantly, as described, in bacteria, the N-terminal methionine is processed off, yielding a homogeneous 153 amino acid form of the protein.

The gene was cloned into a standard procaryotic expression vector between the HindIII and EcoRI restriction sites and transformed into E. coli B. The culture was stored as a glycerol stock or on a plate of L + ampicillin.

A single colony was used to inoculate 50 ml of L-broth containing 50 ug/ml ampicillin and grown overnight at 30°C. 10 ml of this culture was used to inoculate 1 liter of M9 media containing 1 x casamino acids and 50 ug/ml ampicillin. The culture was grown to an optical

density of 0.5 at A_{550} and induced with 50 ug/ml indole acrylic acid as the vector places the gene under control of the trp promoter. The culture was grown overnight at 30°C. In the morning the culture was centrifuged and the pellet was frozen at -78°C until needed.

The pellet was resuspended in 25 ml of 20 mM Na phosphate pH 7, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 0.5 mg/ml lysozyme. After 15 minutes on ice, the suspension was sonicated to rupture the cells. 100 ug each of RNAse and DNAse were added. After 10 minutes on ice, the mixture was centrifuged and the supernatant was saved for purification.

The supernatant was applied to a column of SP-Sephadex (2.5 cm x 2 cm) equilibrated with 20 mM Na phosphate pH 7, 1 mM EDTA. The column was washed with the same buffer until the absorbance at 280 nm returned to baseline levels. The protein was eluted from the column with 20 mM Na phosphate pH 7, 1 mM EDTA, 400 mM NaCl.

The 400 mM NaCl bump from the SP-Sephadex column was loaded onto a column of heparin Sepharose (2.5 cm x 2 cm) equilibrated with 20 mM Tris pH 7.5, 1 mM EDTA, 600 mM NaCl. The column was washed with the same buffer until the absorbance at 280 nm returned to baseline levels. The protein was eluted with 20 mM Na phosphate pH 7.5, 1 mM EDTA, 3 M NaCl.

The protein solution from the heparin Sepharose column was made 10 mM in DTT and incubated 30 minutes on ice, diluted 40 fold with 20 mM Na phosphate pH 7, 1 mM EDTA and repurified on SP-Sephadex as in the first step.

Alternatively, the protein is treated with DTT after the first SP-Sephadex column and then chromatographed on heparin-Sepharose as the final step. This procedure eliminates the need for the second SP-Sephadex column, but leaves the protein in a higher salt buffer.

Example 1

Effect of GSSG Treatment of bFGF

The purified protein from Preparation A, which is the 153 amino acid human sequence of Figure 9 missing met and one ala at the N-terminus, was then added at 0.1 mg/ml to 20 mM Sodium Phosphate buffer pH 7.5 containing 1 mM EDTA. The solution was then made 1 mM in glutathione disulfide and incubated overnight at 4°C. To maximize the conversion to the modified form, after 12 hr the solution was made 4 mM in GSSG, and incubated an additional 24 hr at 4°C. The resulting modified bFGF was purified on SP-Sephadex according to the method of Preparation A (supra).

Figure 1a shows the results of reverse phase HPLC run on a Vydac C_{18} column before treatment as described above, using an acetonitrile/TFA gradient, under conditions shown in the figure. The elution time is between 35 and 40 minutes for the major peak.

After treatment as described above, the elution pattern of Figure 1b is obtained, wherein the major elution peak is at roughly 30 minutes, showing that the treated bFGF is changed in form.

For comparison, Figures 1c and 1d show the elution patterns of an analog of the bFGF, which contains serines rather than cysteines at positions 78 and 96 as shown in the overplaced numbers of Figures 8 and 9. This analog elutes as a single peak at about 33 minutes, and treatment as described above has no effect on the elution pattern. The treatment method thus evidently 1) involves the cysteines at these positions and 2) has no apparent effect on analogs wherein these residues are not present.

Figure 2a shows a further example of the reverse phase HPLC analysis of the bFGF treated as in this Example in comparison with the behavior of material isolated from bovine pituitary by the procedure of Gospodarowicz. Figure 2a shows the HPLC trace for the GSSG-treated recombinant bFGF; Figure 2b shows the corresponding HPLC

trace for the isolated material from pituitary; Figure 2c shows the results of co-injection of the isolated and treated recombinant materials. The behavior of the recombinant bFGF treated with glutathione appears identical with that of the native isolated material.

Finally, Figure 3 shows the activity profiles in the ACE cell proliferation assay for untreated recombinant bFGF of Example 1 and for this bFGF treated with GSSG. No difference in activity is found.

Example 2

Characteristics of GSSG-Treated Recombinant bFGF

Recombinant bFGF treated as described in Example 1 was incubated with 1 mM $CuCl_2$ for 1 min to accelerate, for experimental purposes, the multimerization which ordinarily occurs in untreated recombinant bFGF over time. The resulting bFGF was then subjected to reverse phase HPLC using heparin-TSK. Figures 4a and 4b show that treatment with Cu^{+2} has no effect on the chromatographic pattern of native bFGF isolated from bovine pituitary. However, as shown in Figures 4c and 4d, treatment with Cu^{+2} of recombinant bFGF which has not been stabilized with GSSG results in a multiplicity of peaks and longer retention times.

Figures 4e and 4f show the behavior of recombinant bFGF stabilized with GSSG, which is, like the native form, unaffected by treatment with Cu^{+2}.

The stabilized bFGF prepared in Example 1 is stable over at least a 5-day period, as shown in Figure 5. Figures 5d-5f are a series of HPLC traces derived from chromatography on heparin-TSK performed on the stabilized material. As indicated, in this series of figures, there is no change over the 5-day period. Under the same conditions untreated bFGF forms multimers and shows significant loss of the starting material, Figures 5a-5e.

Example 3

Treatment with Cystine

The procedure of Example 1 was followed except that 1 mM cystine was substituted in the incubation mixture for 1 mM glutathione disulfide and the reaction was performed at pH 7.0. Figure 6a shows the results of reverse-phase HPLC performed on a Vydac C_{18} column with the stabilized bFGF treated in this manner. Figures 6b and 6c show that the cystine-treated bFGF does not precisely co-migrate with the bFGF material isolated from pituitary. Thus the cystine-treated material is apparently not identical in behavior with natural bFGF from bovine pituitaries.

Example 4

bFGF Analogs

The procedure of Example 1 was followed to treat analogs of bFGF with GSSG. The procedure was identical except that the analog having a serine in place of cysteine at position 78 or at position 96 was substituted for the recombinant bFGF. As shown in Figures 7a and 7b, this treatment results in a faster eluting peak in the case of the 78-Ser mutein, thus indicating the effect of GSSG on this analog. Figures 7c and 7d show similar results when the Ser-96 mutein is used (although the reaction is evidently not complete under these conditions).

Claims

1. A method to stabilize basic fibroblast growth factor protein which method comprises:

treating a susceptible peptide having the amino acid sequence of basic fibroblast growth factor (bFGF) with an amount of a protective agent capable of forming a covalent S-S bond with at least one cysteine contained in said susceptible peptide effective to prevent multimerization of said bFGF in the presence of a buffer of pH 6 or greater, said pH at which the bFGF does not denature, for a time sufficient to effect said stabilization.

2. The method of claim 1 wherein the protective agent is a compound of the formula RSSR wherein each R is independently an organic moiety of sufficient hydrophilicity to confer water solubility on said compound.

3. The method of claim 2 wherein the compound of the formula RSSR is selected from the group consisting of glutathione disulfide and cystine.

4. The method of claim 1 wherein the pH is pH 6-8.

5. The method of claim 1 which further includes the step of subjecting the treated bFGF to chromatographic purification.

6. Stabilized basic FGF prepared by the method of claim 1, with the proviso that when bFGF includes the amino acid sequence shown as residues 24-155 of the overplaced numbers shown in Figure 8 or 9, the protective agent is not glutathione disulfide.

7. Stabilized basic FGF prepared by the method of claim 5, with the proviso that when bFGF includes the amino acid sequence shown as residues 24-155 of the overplaced numbers shown in Figure 8 or 9, the protective agent is not glutathione disulfide.

8. A composition of matter which comprises an aqueous medium containing susceptible basic fibroblast growth factor (bFGF) protein along with an amount of a protective agent capable of forming a covalent S-S bond with at least one cysteine contained in said bFGF effective to stabilize said bFGF, said aqueous medium at a pH of at least 6, said pH being such that said bFGF is not denatured.

9. The composition of claim 8 wherein this protective agent is a compound of formula RSSR wherein each R is independently an organic moiety of sufficient hydrophilicity to confer water solubility on said compound.

10. The composition of claim 9 wherein the compound of the formula RSSR is selected from the group consisting of glutathione disulfide and cystine.

Chromatography conditions; (figs. a,b,c,d)
Column; Vydac C_{18} 4.3mm x 25cm 5μm particle size 300Å pore size
Eluents; A: 0.1% TFA B: Acetonitrile + 0.1% TFA
Gradient; 20%B ---------> 40%B ---------> 40%B
40' 5'
Flow; 1ml/min Absorbance; 220nm

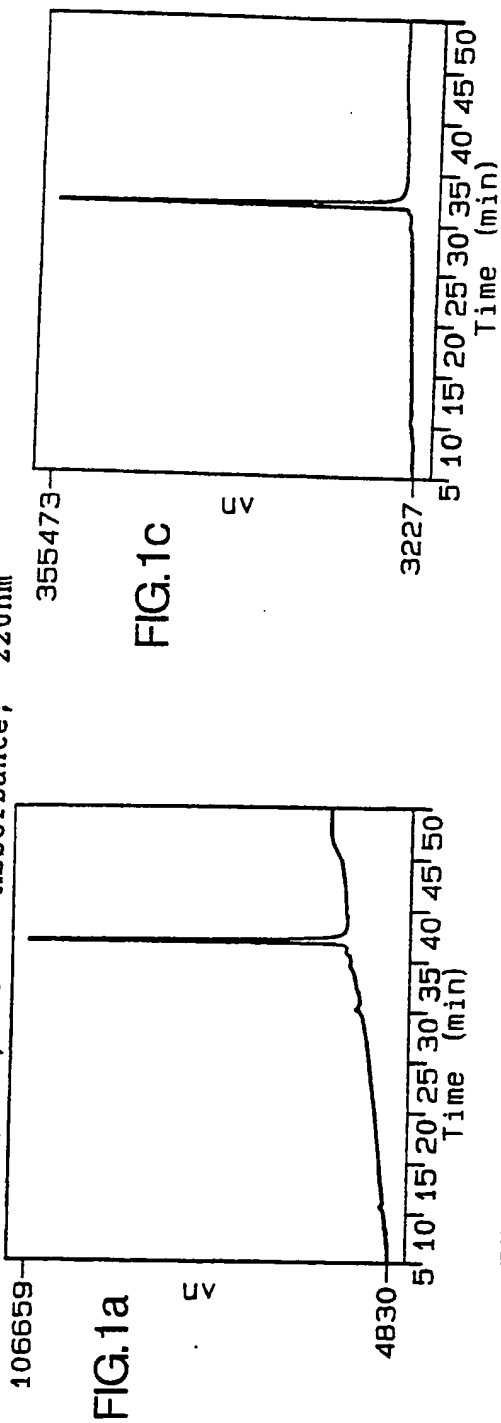


FIG.1a

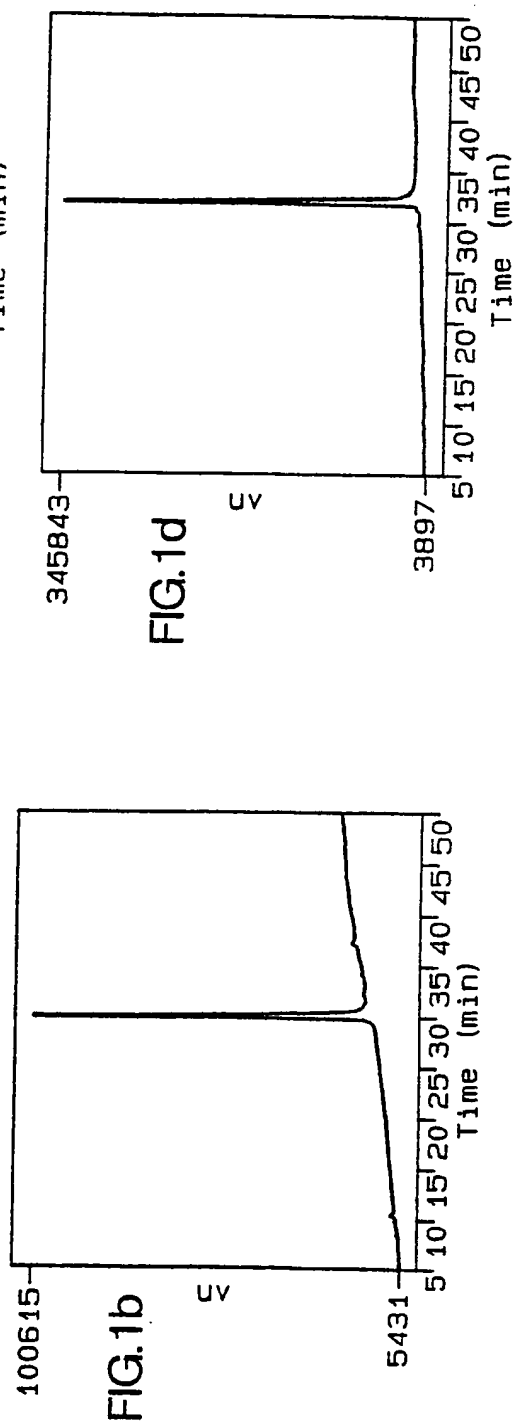


FIG.1b

FIG.1c

FIG.1d

SUBSTITUTE SHEET

Chromatography conditions; (figs. a,b,c)
Column; Vydac C_{18} 4.3mm x 25cm 5µm particle size 300Å pore size
Eluents; A: 0.1% TFA B: Acetonitrile + 0.1% TFA
Gradient; 20%B ---40'---> 40%B ---5'---> 40%B
Flow; 1ml/min Absorbance; 220nm

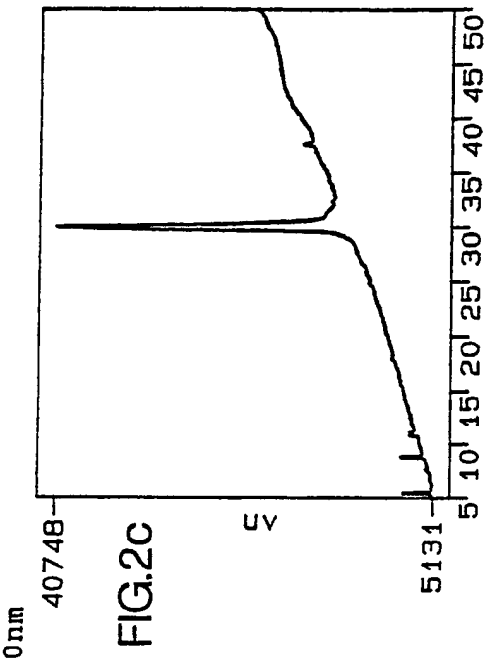


FIG.2c

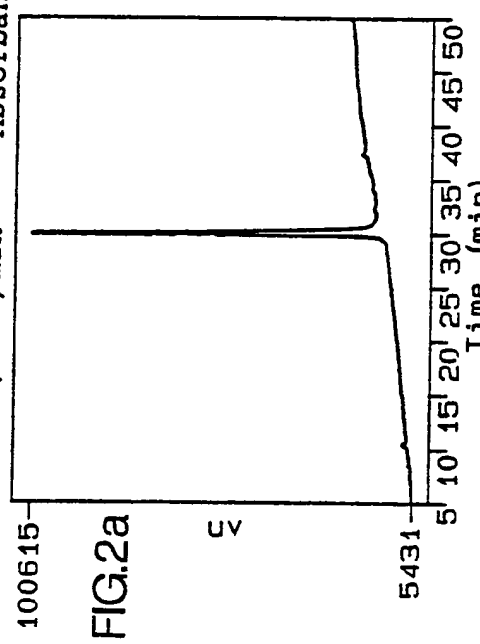


FIG.2a

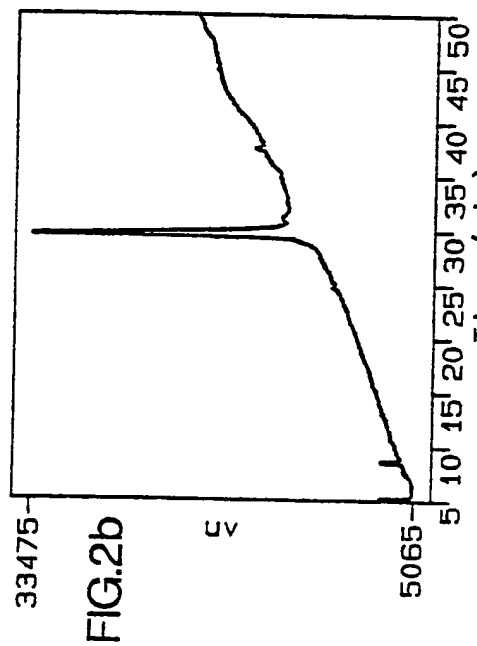
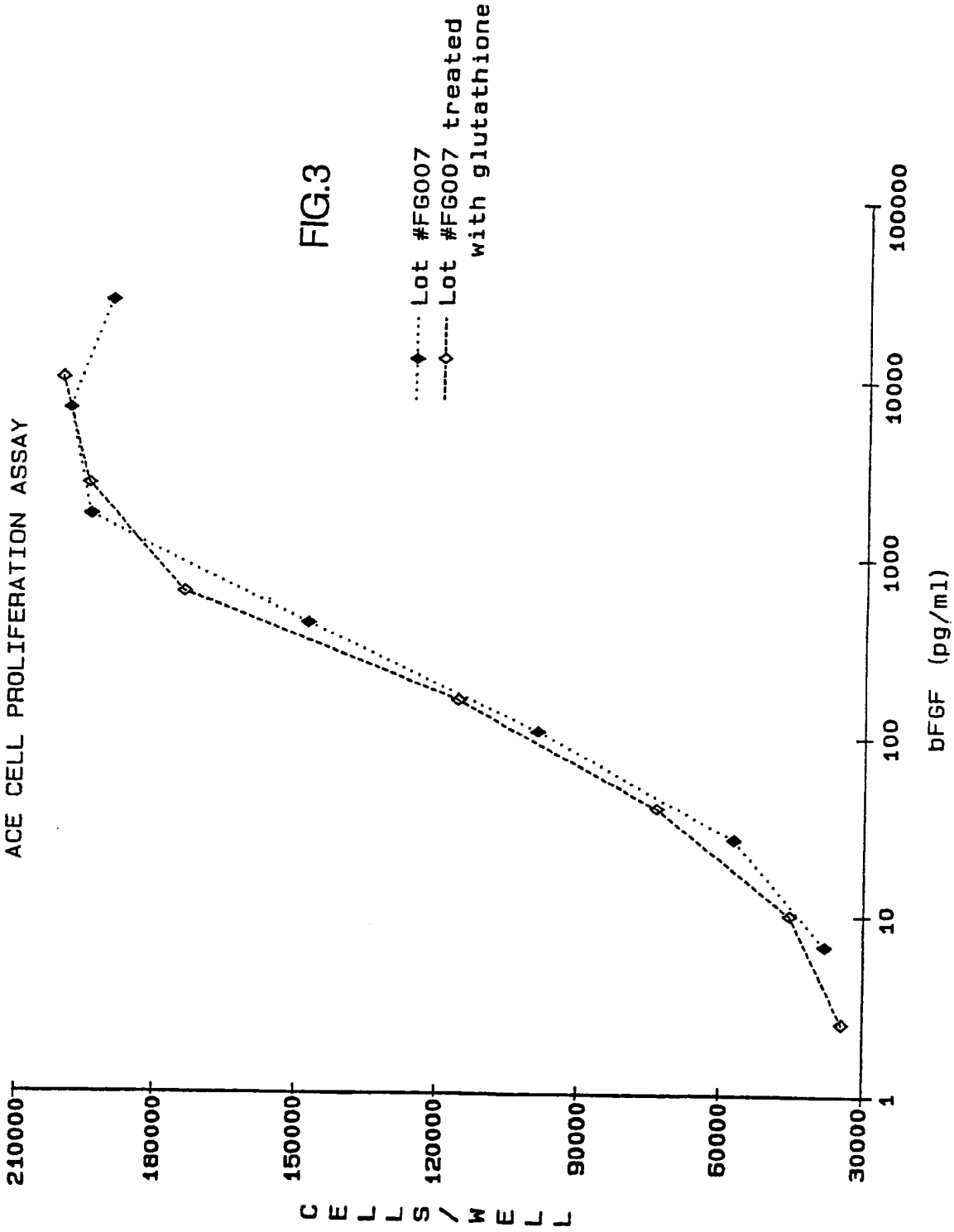


FIG.2b

FIG.3



Chromatography conditions; (figs.a,b,c,d,e,f)

Column; heparin-TSK 7.5mm x 7.5cm

Eluents; A: 20mM Tris pH 7.5 B: 20mM Tris pH 7.5 + 3.0M NaCl

Gradient; 24%B ---1'---> 40%B ---23'---> 100%B ---5'---> 100%B

Flow; 1ml/min Absorbance; 220nm

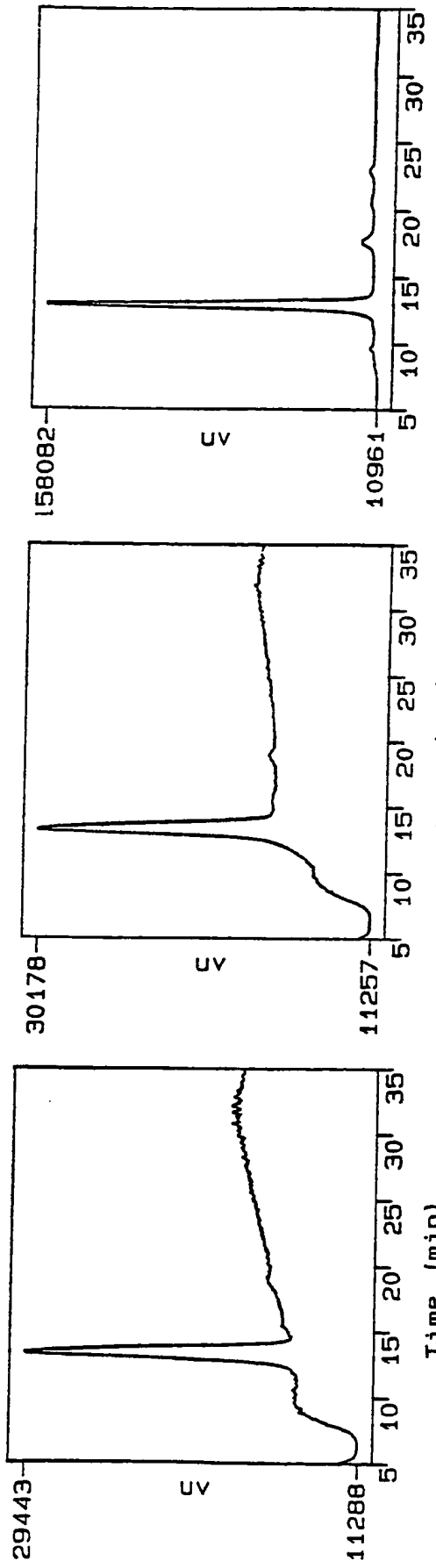


FIG.4a

FIG.4b

FIG.4c

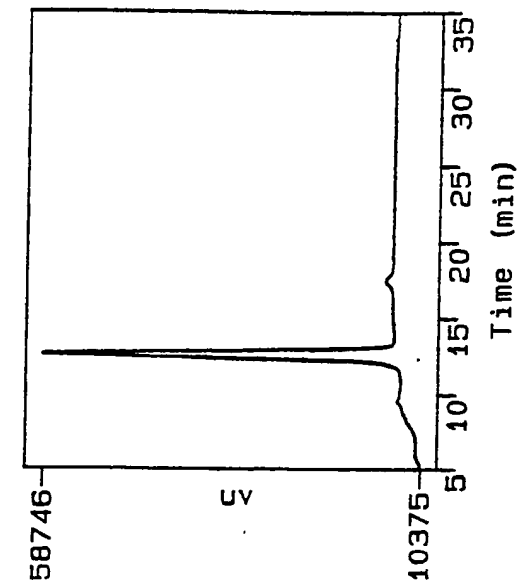


FIG.4f

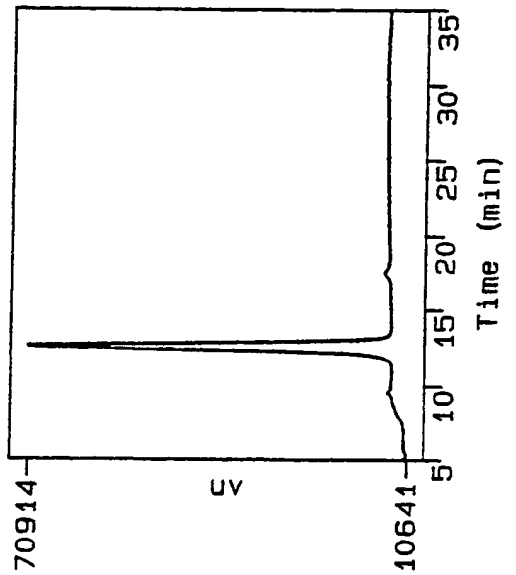


FIG.4e

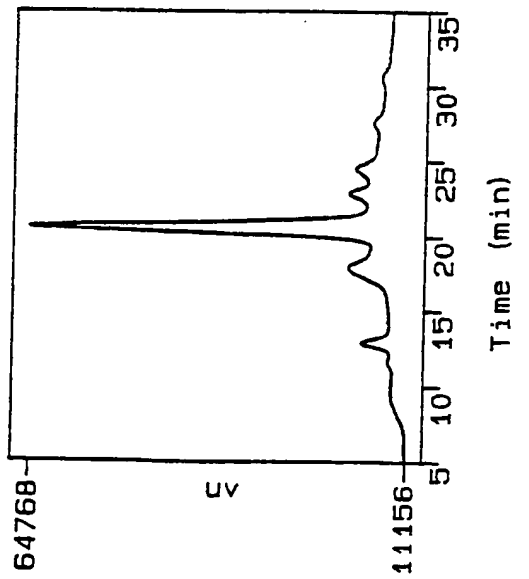


FIG.4d

Chromatography conditions; (figs.a,b,c,d,e,f)

Column; heparin-TSK 7.5mm x 7.5cm

Eluents; A: 20mM Tris pH 7.5 B: 20mM Tris pH 7.5 + 3.0M NaCl

Gradient; 24%B ---1'---> 40%B ---23'---> 100%B ---5'---> 100%B

Flow; 1ml/min Absorbance; 220nm

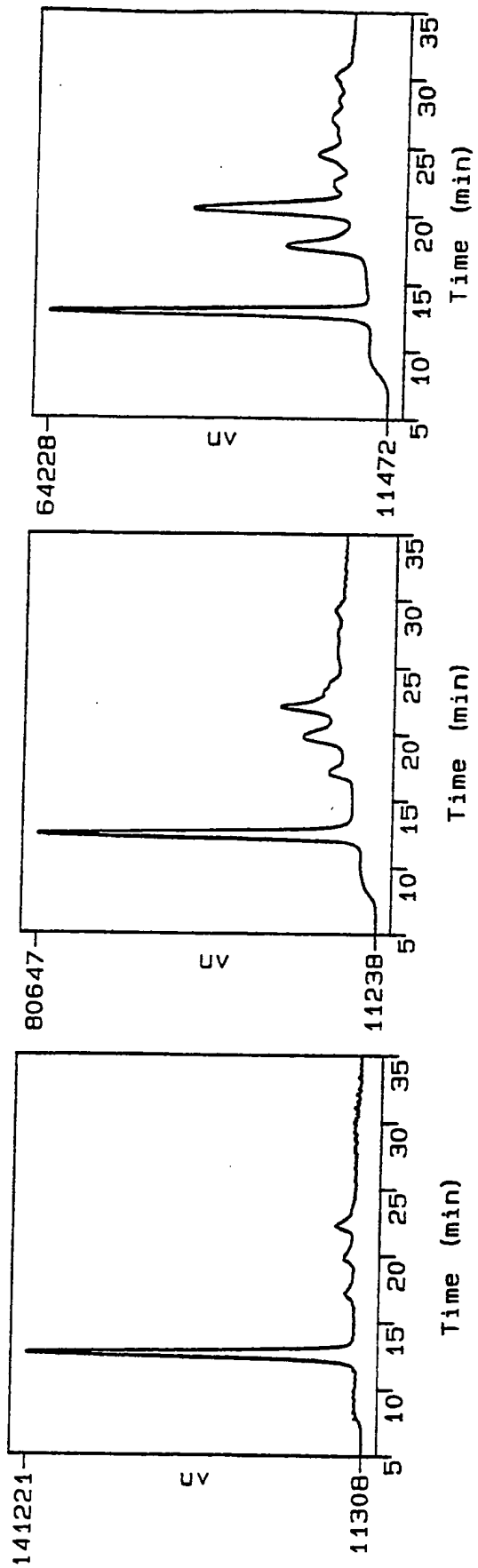


FIG.5a

FIG.5b

FIG.5c

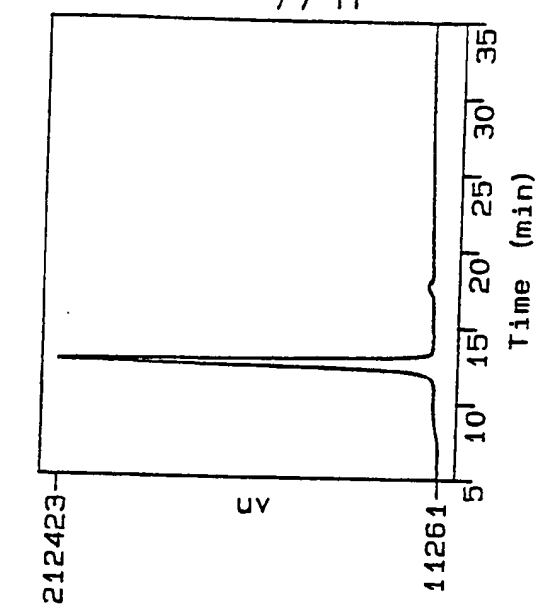


FIG.5f

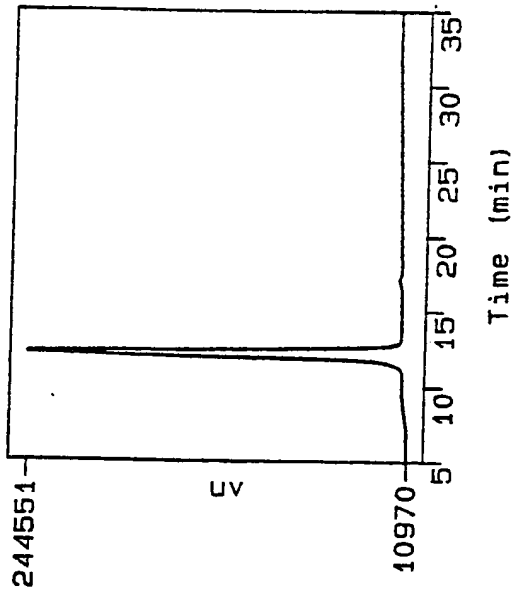


FIG.5e

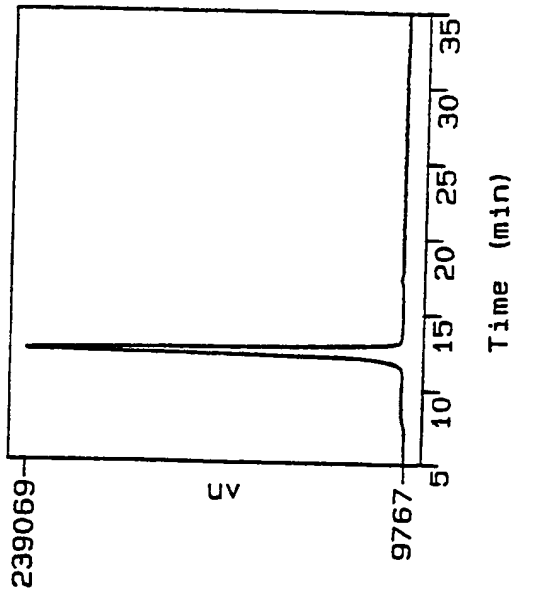


FIG.5d

Chromatography conditions; (figs. a,b)

Column; Vydac C_{18} 4.3mm x 25cm 5μm particle size 300Å pore size

Eluents; A: 0.1% TFA B: Acetonitrile + 0.1% TFA

Gradient; 20%B ---40'---> 40%B ---5'---> 40%B

Flow; 1ml/min Absorbance; 220nm

FIG.6a

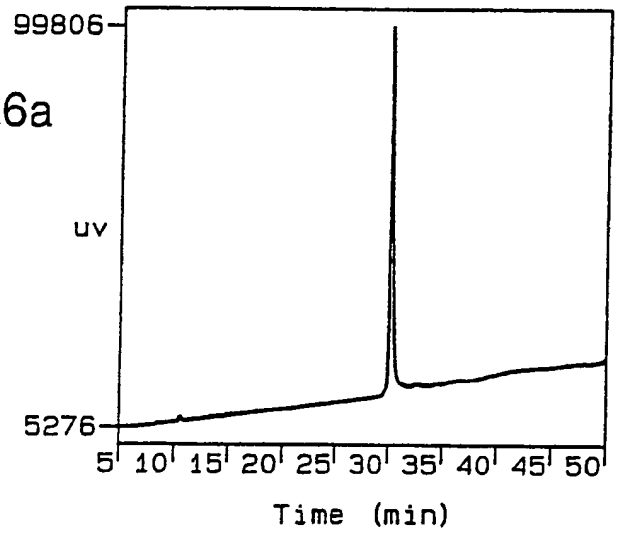
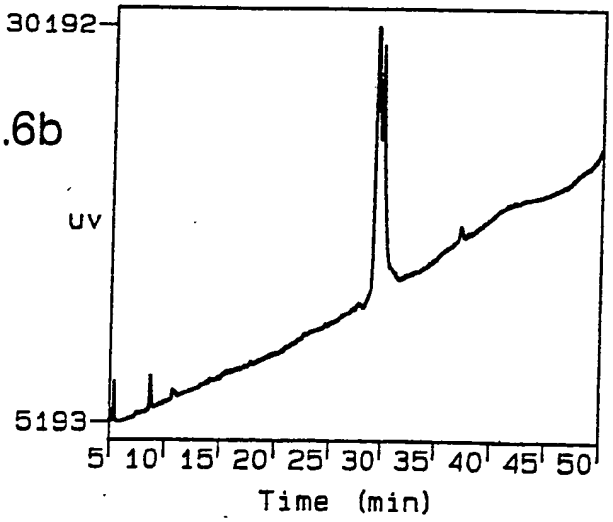


FIG.6b



Chromatography conditions; (figs. a,b,c,d)
Column; Vydac C_{18} 4.3mm x 25cm 5μm particle size 300Å pore size
Eluents; A: 0.1% TFA B: Acetonitrile + 0.1% TFA
Gradient; 20%B ---------> 40%B ---------> 40%B
40' 5'
Flow; 1ml/min Absorbance; 220nm

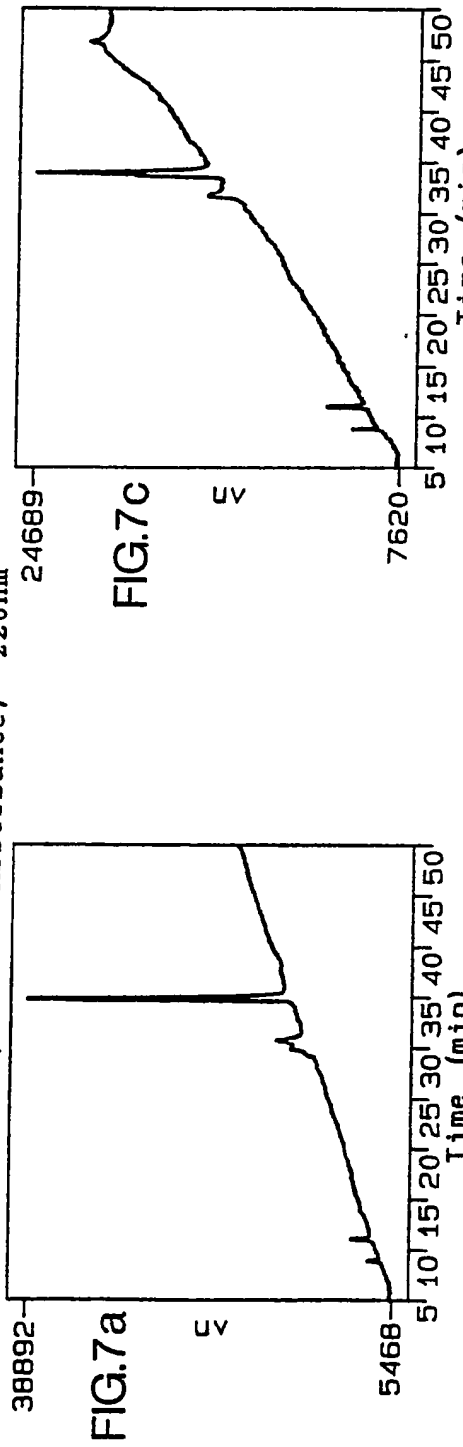


FIG.7a

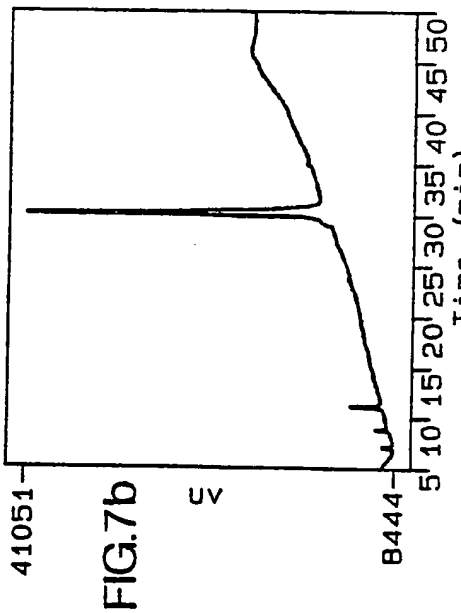


FIG.7b

FIG.7c

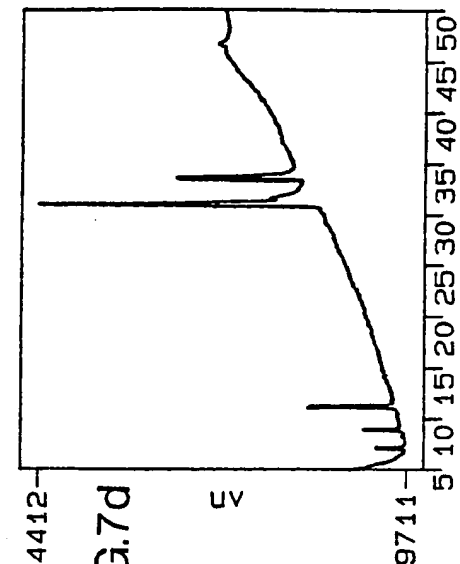


FIG.7d

NUCLEOTIDE SEQUENCE OF THE REGION OF BASIC BOVINE FGF ENCODING AMINO ACIDS 1-146 AND AMINO ACID -1 TO -9

```
                                    1
                                   ATG GCC GCC GGG AGC ATC ACC ACG CTG
                                  (Met Ala Ala Gly Ser Ile Thr Thr Leu)
                                   -9                                -1
  10                                                  20
CCA GCC CTG CCG GAG GAC GGC GGC AGC GGC GCT TTC CCG CCG GGC CAC
Pro Ala Leu Pro Glu Asp Gly Gly Ser Gly Ala Phe Pro Pro Gly His
 1                                                  10
                     30                                          40
TTC AAG GAC CCC AAG CGG CTG TAC TGC AAG AAC GGG GGC TTC TTC CTG
Phe Lys Asp Pro Lys Arg Leu Tyr Cys Lys Asn Gly Gly Phe Phe Leu
                20                                          30
                                         50
CGC ATC CAC CCC GAC GGC CGA GTG GAC GGG GTC CGC GAG AAG AGC GAC
Arg Ile His Pro Asp Gly Arg Val Asp Gly Val Arg Glu Lys Ser Asp
                                    40
          60                                                70
CCA CAC ATC AAA CTA CAA CTT CAA GCA GAA GAG AGA GGG GTT GTG TCT
Pro His Ile Lys Leu Gln Leu Gln Ala Glu Glu Arg Gly Val Val Ser
     50                                                60
                               80
ATC AAA GGA GTG TGT GCA AAC CGT TAC CTT GCT ATG AAA GAA GAT GGA
Ile Lys Gly Val Cys Ala Asn Arg Tyr Leu Ala Met Lys Glu Asp Gly
                          70                                    80
 90                                        100
AGA TTA CTA GCT TCT AAA TGT GTT ACA GAC GAG TGT TTC TTT TTT GAA
Arg Leu Leu Ala Ser Lys Cys Val Thr Asp Glu Cys Phe Phe Phe Glu
                                   90
                110                                         120
CGA TTG GAG TCT AAT AAC TAC AAT ACT TAC CGG TCA AGG AAA TAC TCC
Arg Leu Glu Ser Asn Asn Tyr Asn Thr Tyr Arg Ser Arg Lys Tyr Ser
           100                                         110
                                  130
AGT TGG TAT GTG GCA CTG AAA CGA ACT GGG CAG TAT AAA CTT GGA CCC
Ser Trp Tyr Val Ala Leu Lys Arg Thr Gly Gln Tyr Lys Leu Gly Pro
                               120
      140                                               150
AAA ACA GGA CCT GGG CAG AAA GCT ATA CTT TTT CTT CCA ATG TCT GCT
Lys Thr Gly Pro Gly Gln Lys Ala Ile Leu Phe Leu Pro Met Ser Ala
  130                                   140
  155
AAG AGC TGA TCT TAA
Lys Ser Ter     Ter
  146
```

FIG.8

NUCLEOTIDE SEQUENCE OF THE REGION OF BASIC
HUMAN FGF ENCODING AMINO ACIDS 1-146
AND AMINO ACIDS -1 TO -9

```
                                1
                                ATG GCA GCC GGG AGC ATC ACC ACG CTG
                               (Met Ala Ala Gly Ser Ile Thr Thr Leu)
                                -9                                  -1
 10                                                20
CCC GCC TTG CCC GAG GAT GGC GGC AGC GGC GCC TTC CCG CCC GGC CAC
Pro Ala Leu Pro Glu Asp Gly Gly Ser Gly Ala Phe Pro Pro Gly His
 1                                                10
                    30                                          40
TTC AAG GAC CCC AAG CGG CTG TAC TGC AAA AAC GGG GGC TTC TTC CTG
Phe Lys Asp Pro Lys Arg Leu Tyr Cys Lys Asn Gly Gly Phe Phe Leu
                20                                          30
                                        50
CGC ATC CAC CCC GAC GGC CGA GTT GAC GGG GTC CGG GAG AAG AGC GAC
Arg Ile His Pro Asp Gly Arg Val Asp Gly Val Arg Glu Lys Ser Asp
                                        40
          60                                                70
CCT CAC ATC AAG CTA CAA CTT CAA GCA GAA GAG AGA GGA GTT GTG TCT
Pro His Ile Lys Leu Gln Leu Gln Ala Glu Glu Arg Gly Val Val Ser
      50                                                60
                                80
ATC AAA GGA GTG TGT GCT AAC CGT TAC CTG GCT ATG AAG GAA GAT GGA
Ile Lys Gly Val Cys Ala Asn Arg Tyr Leu Ala Met Lys Glu Asp Gly
                          70                                    80
 90                                                100
AGA TTA CTG GCT TCT AAA TGT GTT ACG GAT GAG TGT TTC TTT TTT GAA
Arg Leu Leu Ala Ser Lys Cys Val Thr Asp Glu Cys Phe Phe Phe Glu
                                                90
                    110                                         120
CGA TTG GAA TCT AAT AAC TAC AAT ACT TAC CGG TCA AGG AAA TAC ACC
Arg Leu Glu Ser Asn Asn Tyr Asn Thr Tyr Arg Ser Arg Lys Tyr Thr
                100                                         110
                                        130
AGT TGG TAT GTG GCA TTG AAA CGA ACT GGG CAG TAT AAA CTT GGA TCC
Ser Trp Tyr Val Ala Leu Lys Arg Thr Gly Gln Tyr Lys Leu Gly Ser
                                        120
          140                                               150
AAA ACA GGA CCT GGG CAG AAA GCT ATA CTT TTT CTT CCA ATG TCT GCT
Lys Thr Gly Pro Gly Gln Lys Ala Ile Leu Phe Leu Pro Met Ser Ala
    130                                             140
    155
AAG AGC TGA TTT TAA
Lys Ser Ter     Ter
    146
```

FIG.9

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/02380

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5) A61K 37/24; A61K 37/36
US Cl. 514/12; 514/970; 514/973

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System	Classification Symbols
US	514/12 514/970 514/973

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 5

APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
X	US, A, 4,816,561 (TODARO) 28 March 1989, see column 27, lines 41-47.	1-10
A	The Journal of Biological Chemistry, Vol. 250, no. 21, issued 10 November 1975, A. Karim Ahmed et al, "Nonenzymatic Reactivation of Reduced Bovine Pancreatic Ribonuclease by Air Oxidation and by Glutathione Oxidation Buffers" See pages 8477-8482.	1-10

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2	Date of Mailing of this International Search Report 2
07 July 1990	24 SEP 1990
International Searching Authority 1	**Signature of Authorized Officer 20**
ISA/US	Fatemeh Moezie

Form PCT/ISA/210 (second sheet) (May 1986)